*DEACYLATED tRNA^{Phe} BINDING TO A RETICULOCYTE RIBOSOMAL SITE FOR THE INITIATION OF POLYPHENYLALANINE SYNTHESIS**

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Abstract.—The initiation of polyphenylalanine synthesis at low $MgCl_2$ concentration in the reticulocyte transfer system has been found to have a stringent requirement for deacylated tRNA^{Phe}. An initial poly-U-directed complex between deacylated tRNA^{Phe} and ribosomes is formed. The onset of polyphenylalanine synthesis causes the rapid release of tRNA^{Phe} from the complex. Thermal dissociation studies indicate that deacylated tRNA^{Phe} and phenylalanyl-tRNA are bound to the same ribosomes. A total of three ribosomal tRNA binding sites is indicated.

Introduction.—Reports from several laboratories indicate that *N*-formylmethionyl-tRNA is not involved in the initiation of globin peptide chains in rabbit reticulocytes.[1–4] Furthermore, incorporation of *N*-acetylphenylalanine into the *N*-terminal position of polyphenylalanine synthesized in the poly-U-directed reticulocyte transfer system could not be demonstrated.[15] This is in contrast to the observation that *N*-acetylphenylalanine is incorporated into polyphenylalanine in transfer systems derived from *E. coli*.[5, 6] Earlier work in this laboratory indicated that deacylated tRNA^{Phe} is involved in initiation of polyphenylalanine synthesis in the reticulocyte transfer system.[4, 8, 15] The initial reactions involve very small amounts of tRNA^{Phe} that appear to be approximately equal on a molar basis to the number of ribosomes that are capable of the subsequent synthesis of polyphenylalanine. These initial reactions in the reticulocyte transfer system do not require GTP and thus appear to differ from the reactions by which *N*-acetylphenylalanyl-tRNA or *N*-formylmethionyl-tRNA are bound to *E. coli* ribosomes.[6, 7] These observations may reflect basic differences in the mechanism by which peptides are initiated in bacteria and higher organisms, at least in *E. coli* and rabbit reticulocytes.

The ability of deacylated tRNA^{Phe} to participate in the early reactions of polyphenylalanine synthesis in the reticulocyte system is destroyed by hydrolytic removal of the 3′-terminal adenosine[8] or by periodate oxidation of the 3′-terminal ribose of its adenosine moiety.[4] Takanami[9] and Cannon, Krug, and Gilbert[10] have observed binding of deacylated tRNA to *E. coli* ribosomes that was not dependent upon an energy source or aminoacyl-tRNA but that was dependent upon the integrity of the -pCpCpA end of the tRNA molecule.

The experiments reported here indicate that in the reticulocyte transfer system, polyphenylalanine synthesis at a Mg^{++} concentration of 8 mM or below is highly dependent upon deacylated tRNA^{Phe}. The formation of a ribosomal complex with poly U and deacylated tRNA^{Phe} is involved. The release of deacylated tRNA from the complex shows an absolute requirement for all the components involved in peptide bond synthesis. The relation between peptide

bond formation, translation, and the ribosomal sites involved in binding phenylalanyl-tRNA and deacylated $tRNA^{Phe}$ is discussed.

Materials and Methods.—The preparation of ribosomes, phenylalanyl-tRNA, crude transfer enzyme fractions,[11] deacylated tRNA,[4, 12] and periodate-treated phenylalanyl-tRNA[4] has been described previously. Manuscripts describing the purification of the binding enzyme TF-I and the second transfer factor TF-II, previously designated as peptide synthetase, are in preparation. The *N*-ethylmaleimide inactivation of TF-II bound to ribosomes and present in the crude soluble enzyme preparations has been described previously.[13]

Assay conditions: The preliminary incubation mixture contained the following components in a final volume of 0.25 ml: 0.06 *M* Tris-HCl, pH 7.5; 0.07 *M* KCl; 0.008 *M* MgCl; 0.01 *M* GSH; 100 μg of poly U; 250 μg of ribosomes; and the indicated amount of deacylated tRNA. A second incubation was carried out in a final volume of 0.50 ml containing Tris-HCl (pH 7.5), KCl, and GSH at the same concentration as the preliminary incubation mixture. The $MgCl_2$ concentration in the second incubation mixture was 4 mM and contained 70 $\mu\mu$moles ^{14}C-phenylalanyl-tRNA (about 100 μg tRNA, 2800 cpm). Incubations performed with crude transfer enzymes contained about 120 μg of protein. Incubation mixtures in which fractionated enzymes were used contained 5 μg binding enzyme protein and 3.1 μg TF-II protein. Polymerization reaction mixtures contained 0.1 μmole GTP and were incubated for the time indicated in the figure legends.

Fractionated deacylated $tRNA^{Phe}$ and tRNA deficient in phenylalanine acceptance were obtained from the National Institute of General Medical Sciences and Dr. G. D. Novelli. This material was labeled by Dr. J. M. Clark, Jr., employing the procedure developed by Shelton and Clark.[14] This material contained in excess of 20,000 dpm/μg.

Determination of ^3H·tRNA bound to ribosomes: Binding of tRNA to the ribosomes was measured by filtering the assay mixture through a Millipore filter. The filters were then placed in scintillation vials, and 0.40 ml of 0.1 *N* NaOH was added. The vials were heated to 100° for 10 min to solubilize the bound tRNA. After being cooled, the samples were neutralized by the addition of 0.05 ml of 1 *N* acetic acid and then counted in a Beckman 150 scintillation counter in a counting mixture of 0.5% 2,5-diphenyloxazole in toluene containing 10% Biosolv[3] (Beckman Instruments, Inc.).

Results.—The requirement for deacylated tRNA for polyphenylalanine synthesis is depicted in Figure 1. Without preliminary incubation the Mg^{++} optimum for polymerization is 8 mM, and preparations of unfractionated tRNA charged with phenylalanine form polyphenylalanine relatively slowly, reaching

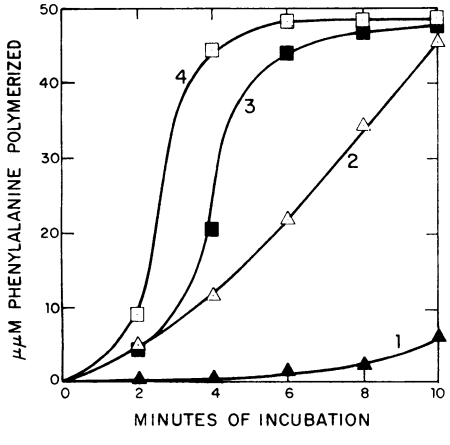

FIG. 1.—The effect of preliminary incubation on polyphenylalanine synthesis at 4 mM Mg^{++} with untreated and periodate-treated phenylalanyl-tRNA. Ribosomes and poly U were preincubated in the presence or absence of 5 μg of deacylated tRNA under the conditions described in *Materials and Methods*. Following preincubation the components required for polymerization were added and the samples incubated for the indicated time.

1, No tRNA in preincubation, periodate-treated phenylalanyl-tRNA in the incubation.

2, No tRNA in preincubation, untreated phenylalanyl-tRNA in incubation.

3, 5 μg tRNA in preincubation, periodate-treated phenylalanyl-tRNA in the incubation.

4, 5 μg tRNA in preincubation, untreated phenylalanyl-tRNA in the incubation.

maximum levels of incorporation after about ten minutes of incubation. Preliminary incubation of the reaction mixture at 8 mM Mg^{++} before the addition of transfer enzymes and GTP shifts the Mg^{++} optimum for polymerization during a second incubation to 4 mM, and polyphenylalanine synthesis proceeds rapidly, giving maximum incorporation in about four minutes. This difference in the rate of polyphenylalanine synthesis at 4 mM $MgCl_2$ forms the basis of the Mg^{++} shift assay system described previously.[4, 8]

Sodium metaperiodate treatment of preparations of tRNA acylated with phenylalanine greatly reduces the capacity of the preparations to support polyphenylalanine synthesis at 4 mM $MgCl_2$. The inability of periodate-treated phenylalanyl-tRNA to support the synthesis of polyphenylalanine is due to the inactivation of the deacylated $tRNA^{Phe}$ present in the preparation.[4] As a result, synthesis of hot trichloroacetic acid-insoluble polyphenylalanine is not detectable until after approximately six or seven minutes of incubation. The onset of synthesis using these periodate-treated preparations probably requires the hydrolysis of small amounts of phenylalanyl-tRNA to form deacylated $tRNA^{Phe}$ during the course of the incubation. In support of this hypothesis, preliminary incubation with very small amounts of deacylated tRNA, poly U, and ribosomes almost completely restores the activity of the oxidized preparation of phenylalanyl-tRNA. Thus we interpret these results as indicating a stringent, possibly absolute, requirement for intact deacylated $tRNA^{Phe}$ for the initiation of polyphenylalanine synthesis in the reticulocyte transfer system.

Earlier work demonstrated that a very small amount of $tRNA^{Phe}$ was required during preliminary incubation to give maximum amounts of polyphenylalanine synthesis in the Mg^{++} shift assay system.[4, 8, 15] The molar ratio of $tRNA^{Phe}$ to ribosomes appeared to be in the order of 0.1. This low ratio was interpreted to reflect a specific, codon-directed interaction of deacylated $tRNA^{Phe}$ with active ribosomes capable of the subsequent synthesis of polyphenylalanine. However, alternative explanations could not be eliminated, such as an interaction of $tRNA^{Phe}$ with a potentially soluble enzyme present in ribosome preparations. The experiments represented in Figure 2 provide direct evidence for the formation of a ribosomal complex involving poly U and deacylated $tRNA^{Phe}$.

Two fractions of *E. coli* tRNA were labeled by the tritium exchange procedure developed by Shelton and Clark.[14] One fraction contained relatively pure $tRNA^{Phe}$. The other contained the bulk of the tRNA and had low but detectable acceptance capacity for phenylalanine.

The labeled tRNA fractions were incubated with ribosomes and poly U, if indicated, under the conditions of the Mg^{++} shift preliminary incubation, and quickly chilled. A relatively large amount of unlabeled, unfractionated rabbit liver tRNA was added, and the tubes were reheated for five minutes at the indicated temperature. Labeled tRNA that remained bound to the ribosomes after the second incubation in which exchange of nonspecifically bound tRNA was carried out was determined by filtering the reaction mixture through a Millipore filter.

The effect of poly U on the binding stability of deacylated $H^3 \cdot tRNA^{Phe}$ to ribosomes is shown in Figure 2*A*. When poly U is not present in the system,

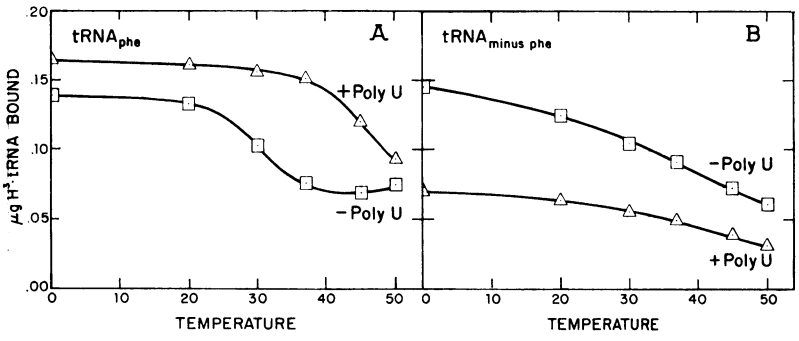

FIG. 2.—The effect of poly U on the binding of deacylated tRNA to ribosomes. The samples were preincubated in the presence of 1 μg of labeled, fractionated tRNA as described in *Materials and Methods*. After the preliminary incubation the samples were chilled, and 50 μg of unlabeled, unfractionated, deacylated tRNA were added. The samples were then incubated for 5 min at the indicated temperature. Following incubation the assays were diluted rapidly with cold buffer, filtered, and counted as described in *Materials and Methods*. Figure 2*A* shows the results of this procedure with H^3·tRNA^{Phe}. The results with the labeled tRNA fraction deficient in tRNA^{Phe} (tRNA^{-Phe}) are shown in Figure 2*B*. (□—□—□, no poly U; △—△—△, poly U added.)

more than 50 per cent of the total amount of labeled tRNA^{Phe} bound to the ribosomes at 25° is lost by heating to 37° in the presence of a relatively large amount of unlabeled tRNA. Under these conditions heating the reaction mixture to 50° causes no further loss of H^3·tRNA^{Phe}. Half of the H^3·tRNA^{Phe} bound at 25° but lost above 37° is lost by heating to 31°. We refer to this as the temperature of half maximum loss. Addition of poly U to the preliminary incubation mixture stabilizes the ribosome-H^3·tRNA^{Phe} complex so that a second incubation at 37° in the presence of an excess amount of unfractionated, unlabeled tRNA causes little or no loss of labeled tRNA^{Phe} from the ribosomes. Half maximum loss of the tRNA^{Phe} bound in response to the poly U occurs at 45°. We interpret these data to indicate the formation of a specific, codon-directed complex between ribosomes, poly U, and deacylated tRNA^{Phe}.

The corresponding experiments with the labeled tRNA fraction deficient in tRNA^{Phe} (tRNA^{-Phe}) are depicted in Figure 2*B*. When poly U is not present, the amount of this tRNA bound corresponds closely to the level of tRNA^{Phe} bound in the absence of poly U. Addition of poly U causes a decrease in the amount of tRNA^{-Phe} bound at all temperatures.

It is of particular interest to identify the ribosomal site involved in binding deacylated tRNA. Data bearing on this problem are provided by the experiments presented in Table 1 that demonstrate the effect of transfer enzymes and GTP on the complex involving deacylated tRNA^{Phe}. For these experiments, ribosomes, poly U, and deacylated tRNA^{Phe} were incubated to form a ribosomal complex. Unlabeled tRNA charged with ^{14}C-phenylalanine was then added, and the incubation continued for five minutes at 37° as described above. These conditions provide a near-maximum net amount of the specific ribosome-tRNA^{Phe} complex formed in response to poly U. The components indicated in Table 1 were added to these reaction mixtures. GTP, binding enzyme, or TF-II alone

TABLE 1. *The effect of transfer factors on the deacylated H^3-tRNA^{Phe}-ribosome complex.*

Additions to incubation*	Bound deacylated tRNA^{Phe} (μg)
None	0.079
Deacylated tRNA	0.080
Binding enzyme	0.077
Binding enzyme + GTP	0.078
TF-II	0.079
TF-II + GTP	0.076
Binding enzyme + TF-II	0.081
Binding enzyme + TF-II + GTP	0.062
Crude enzyme	0.079
Crude enzyme + GTP	0.033
NEM† crude Enzyme + GTP	0.078

The effect of the addition of transfer factors on the binding of tRNA^{phe} to ribosomes. Labeled deacylated tRNA^{phe} was bound to ribosomes during a preliminary incubation. After the samples were chilled, 100 μg of tRNA charged with phenylalanine was added to the preliminary incubation mixture, and the samples were incubated at 37° for 5 min to allow exchange of nonspecifically bound tRNA to occur. The samples were chilled, and the indicated components were added. Incubation was then carried out for 2 min. The samples were diluted rapidly with cold buffer, filtered, and counted as described in *Materials and Methods*. The amount of H^3-tRNA^{phe} remaining on ribosomes in control reaction mixtures lacking poly U was subtracted from the values listed. This amount was about 0.075 μg for all reaction conditions.

* Phenylalanyl-tRNA added to all.

† NEM, *N*-ethylmaleimide treated to inactivate TF-II.

or in any combination of two had no apparent effect in displacing deacylated tRNA^{Phe} bound in response to poly U from the ribosomal complex. Without added enzyme or GTP, about 3 μμmoles of phenylalanyl-tRNA are nonenzymatically bound to the ribosomes. Larger amounts of nonenzymatic binding are observed at higher concentrations of Mg^{++} but appear to have no effect in reducing the amount of poly U-directed deacylated tRNA^{Phe} bound to the ribosomes. The combination of GTP and binding enzyme supports the GTP-dependent enzymatic binding of about 13 μμmoles of phenylalanyl-tRNA to the ribosomes with no reduction in the amount of tRNA^{Phe} bound to the complex. These experiments suggest that nonenzymatically and enzymatically bound phenylalanyl-tRNA occupy sites other than the site in which the deacylated tRNA^{Phe} is bound. This hypothesis is presented below.

As shown in Table 1, when GTP, binding enzyme, and TF-II fraction are present in the reaction mixture, deacylated tRNA^{Phe} bound in response to poly U is lost from the complex. The time course for this reaction is presented in Figure 3 and indicates a very rapid loss of a portion of the tRNA^{Phe} present in the complex. The loss of tRNA^{Phe} appears to be coincident with the onset of polyphenylalanine synthesis.

With a crude enzyme fraction the amount of tRNA^{Phe} lost from the ribosomes during the initial ten-second period of peptide synthesis is about 65 per cent of the tRNA^{Phe} bound to the ribosomes in response to poly U. The use of highly purified transfer factors causes the release of 20–25 per cent of the tRNA^{Phe} bound to the ribosomes. The reason for the increased effectiveness of the crude enzyme is not clear but may be related to the presence of a chain initiation factor reported previously.[12]

The hypothesis that deacylated tRNA^{Phe} and enzymatically or nonenzymatically bound phenylalanyl-tRNA occupy different ribosomal sites is further sup-

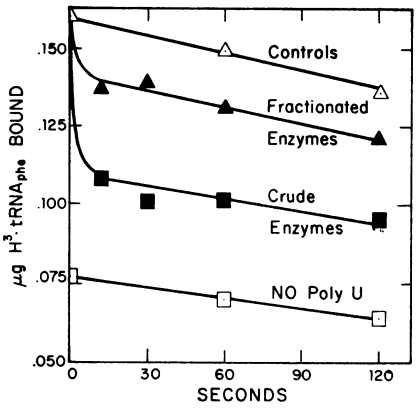

FIG. 3.—The effect of polyphenylalanine synthesis on the binding of deacylated tRNA^{Phe} to ribosomes. Binding of tRNA to ribosomes and exchange of nonspecifically bound tRNA were carried out as described in the legend to Table 1. The samples were chilled and the indicated enzyme fractions added. Following a 60-sec incubation at 37° to bring the samples to temperature, the reaction was started by adding 0.1 μmole of GTP. Control experiments received an equal volume of water. At the indicated time following the addition of GTP the samples were diluted rapidly with cold buffer and filtered.

—△—, control. No factor nor any of the following combinations of factors, was added: binding enzyme + GTP, *N*-ethylmaleimide-treated crude enzyme + GTP, TF-II + GTP, binding enzyme + TF-II.

—▲—, binding enzyme + TF-II + GTP.

—■—, crude enzyme fraction + GTP.

—□—, no poly U in preliminary or final incubation.

ported by the data presented in Figure 4. For these experiments poly U-directed binding of deacylated tRNA^{Phe} was carried out as described for Figure 3. Following the binding of deacylated tRNA, phenylalanyl-tRNA was bound to the ribosomes by either the nonenzymatic or enzymatic mechanisms. Enzymatic binding was carried out in a subsequent incubation following the addition of phenylalanyl-tRNA, binding enzyme, and GTP. The final $MgCl_2$ concentration of the reaction mixture was 8 mM. Nonenzymatic binding was carried out in an identical manner, except that binding enzyme and GTP were omitted from the reaction mixture. The effect of these reactions on the heat stability of the deacylated tRNA^{Phe}-ribosome complex is shown in Figure 4. There is an in-

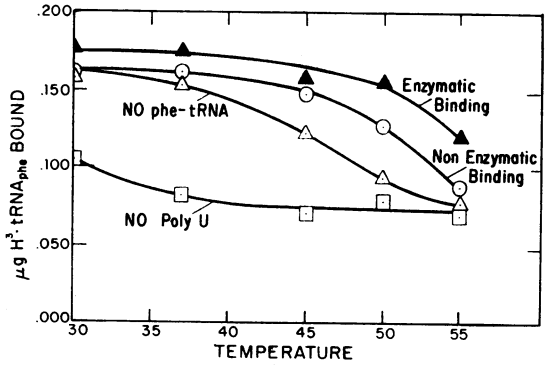

FIG. 4.—Effect of phenylalanyl-tRNA binding on the heat stability of the deacylated tRNA^{Phe}-ribosome complex. Binding of labeled deacylated tRNA^{Phe} was carried out during an 8-min preliminary incubation. After this preincubation the components indicated below were added; then the reaction mixtures were incubated for an additional 8 min. □—□—□, no poly U control; △—△—△, unfractionated deacylated tRNA; ○—○—○, phenylalanyl-tRNA; ▲—▲—▲, phenylalanyl-tRNA + binding enzyme + GTP.

crease in the stability of the binding of deacylated $tRNA^{Phe}$ to the ribosome when nonenzymatic or enzymatic binding is carried out. Other experiments have demonstrated that enzymatically bound phenylalanyl-tRNA and $tRNA^{Phe}$ are lost from the ribosome complex at about the same temperature. Under these conditions loss of half of the enzymatically bound phenylalanyl-tRNA occurs at about 56°. Considered together, these data indicate that deacylated $tRNA^{Phe}$ and enzymatically bound phenylalanyl-tRNA can be simultaneously bound to the same ribosome to form a relatively stable complex.

Discussion.—We find that deacylated tRNA functions as an initiator of polyphenylalanine synthesis in the poly U-directed reticulocyte transfer system. The evidence suggests that the requirement for deacylated tRNA may be absolute, at least at relatively low Mg^{++} concentrations (8 mM or below) that support efficient synthesis in this system. The data provide support for our earlier hypothesis that the initial steps in the synthesis of polyphenylalanine in the reticulocyte system involve the formation of a complex between ribosomes, poly U, and deacylated $tRNA^{Phe}$. It appears that a codon-anticodon interaction between poly U and deacylated $tRNA^{Phe}$ may be involved, as well as a reaction requiring the deacylated $tRNA^{Phe}$ to have an intact 3′ (pCpCpA) terminus.

Diphenylalanine can be formed in the presence of TF-II and GTP from phenylalanyl-tRNA originally bound to ribosomes during the enzymatic binding reaction. These data indicate that a ribosome may bind two molecules of phenylalanyl-tRNA. Furthermore, as described here, deacylated tRNA is stringently required for polyphenylalanine synthesis in a codon-directed reaction in which it is also bound to ribosomes. Three ribosomal sites capable of binding tRNA appear to be indicated.

We reported earlier that binding of deacylated $tRNA^{Phe}$ appears to involve the larger ribosomal subunit and suggested that this may be at the peptidyl site.[8] Under the conditions used here, deacylated tRNA is efficiently displaced from the ribosomal complex only in the presence of TF-II and the binding enzyme. We interpret these results to indicate that a ribosomal site adjacent in the reaction sequence to the site to which deacylated tRNA is bound is filled with phenylalanyl-tRNA by the enzymatic binding reaction. Deacylated tRNA is then displaced from the ribosomal complex by the action of TF-II in translocation of enzymatically bound phenylalanyl-tRNA into the peptidyl site. Considering this and results which indicate that GTP hydrolysis may be involved with reactions catalyzed by both the binding enzyme and TF-II,[16] we believe that peptide bond formation on reticulocyte ribosomes may involve three ribosomal sites through which tRNA is moved in two independent reactions each requiring GTP hydrolysis.

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